Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Stem Cell Line

Generation of induced pluripotent stem cells from Chinese hamster embryonic fibroblasts

Haiyun Pei [a,b,c,1], Hsu-Yuan Fu [a,1], Hiroyuki Hirai [d], Dong Seong Cho [a,b], Timothy D. O'Brien [b,f], James Dutton [b], Catherine M. Verfaillie [e], Wei-Shou Hu [a,b,*]

[a] Department of Chemical Engineering and Materials Science, Minneapolis, MN, USA
[b] Stem Cell Institute, University of Minnesota, Minneapolis, MN, USA
[c] Stem Cell and Regenerative Medicine Lab, Beijing Institute of Transfusion Medicine, Beijing, China
[d] Department of Cardiac Surgery, University of Michigan, Ann Arbor, MI, USA
[e] Department of Development and Regeneration, Katholieke Universiteit Leuven, Leuven, Belgium
[f] Veterinary Population Medicine Department, University of Minnesota, St. Paul, MN, USA

ARTICLE INFO

Article history:
Received 28 March 2017
Received in revised form 13 April 2017
Accepted 19 April 2017
Available online 21 April 2017

ABSTRACT

We derived a stable cell line from Chinese hamster embryonic fibroblasts by transduction of four mouse transcription factors (*M_3O*, *Sox2*, *Klf4*, and *n-Myc*) using a lentiviral vector. The cell line possess all the characteristics of an induced pluripotent stem cell (iPSC) line. Given that Chinese hamster ovary (CHO) cells are the predominant host cells used for therapeutic protein production and no pluripotent stem cell line or other normal cell line has been isolated from Chinese hamster, this iPSC line may serve as a useful tool for research using CHO cells or even be used for deriving new cell lines.

Resource table

Name of stem cell line	CHiPSC-B
Institution	University of Minnesota
Contact information of distributor	Wei-Shou Hu, acre@umn.edu
Type of cell line	Inducible pluripoten stem (iPS) cell
Origin	Chinese hamster (*Cricetulus griseus*)
Cell Source	Chinese hamster embryonic fibroblasts
Method of reprogramming	Lentivirus
Name of transgene or resistance	M_3O, Sox2, Klf4, n-Myc
Inducible/constitutive system	CMV promoter, pLOVE system
Date archived/stock date	2014–2015

Resource utility

The work reported here can be applied to producing genetically modified Chinese hamster or offer a template for establishing Chinese hamster ESCs. The cell line can serve as the normal diploid cell reference for exploring genome engineering of CHO cells, and has an immediate impact on biomanufacturing of therapeutic proteins.

Resource details

Recombinant cell lines derived from CHO cells are used for the production of the majority of therapeutic proteins, including monoclonal antibodies, erythropoietin, Factor VIII. In spite of their industrial and scientific importance, no Chinese hamster induced pluripotent stem cells (CHiPSCs) have been established. Since no Chinese hamster embryonic stem cell (ESC) line has been isolated, the culture conditions for maintaining pluripotent state are not known.

Chinese hamster embryonic fibroblasts (CHEFs) (Fig. 1A) were transduced with the lentiviral vector cocktail of the mouse transcription factors M3O (Hirai et al., 2011), Sox2, Klf4, and n-Myc (*SKM*). Colonies with an ESC-like morphology first became visible around 6 days after transduction. Between 100 and 200 colonies emerged eventually from 10^5 cells plated in a well. The isolated ESC-like cells (CHiPSCs-B) were similar to mouse ESCs in morphology at the individual cell as well as the colony level. They had smooth, refractile defined colony borders and a compact internal architecture comprised of small, tightly packed round cells (Fig. 1B). CHiPSCs-B was positive for *Nanog* promoter-GFP reporter assay

* Corresponding author at: Department of Chemical Engineering and Materials Science, University of Minnesota, 421 Washington Avenue SE, Minneapolis, MN 55455-0132, USA.
E-mail address: acre@cems.umn.edu (W.-S. Hu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.scr.2017.04.010

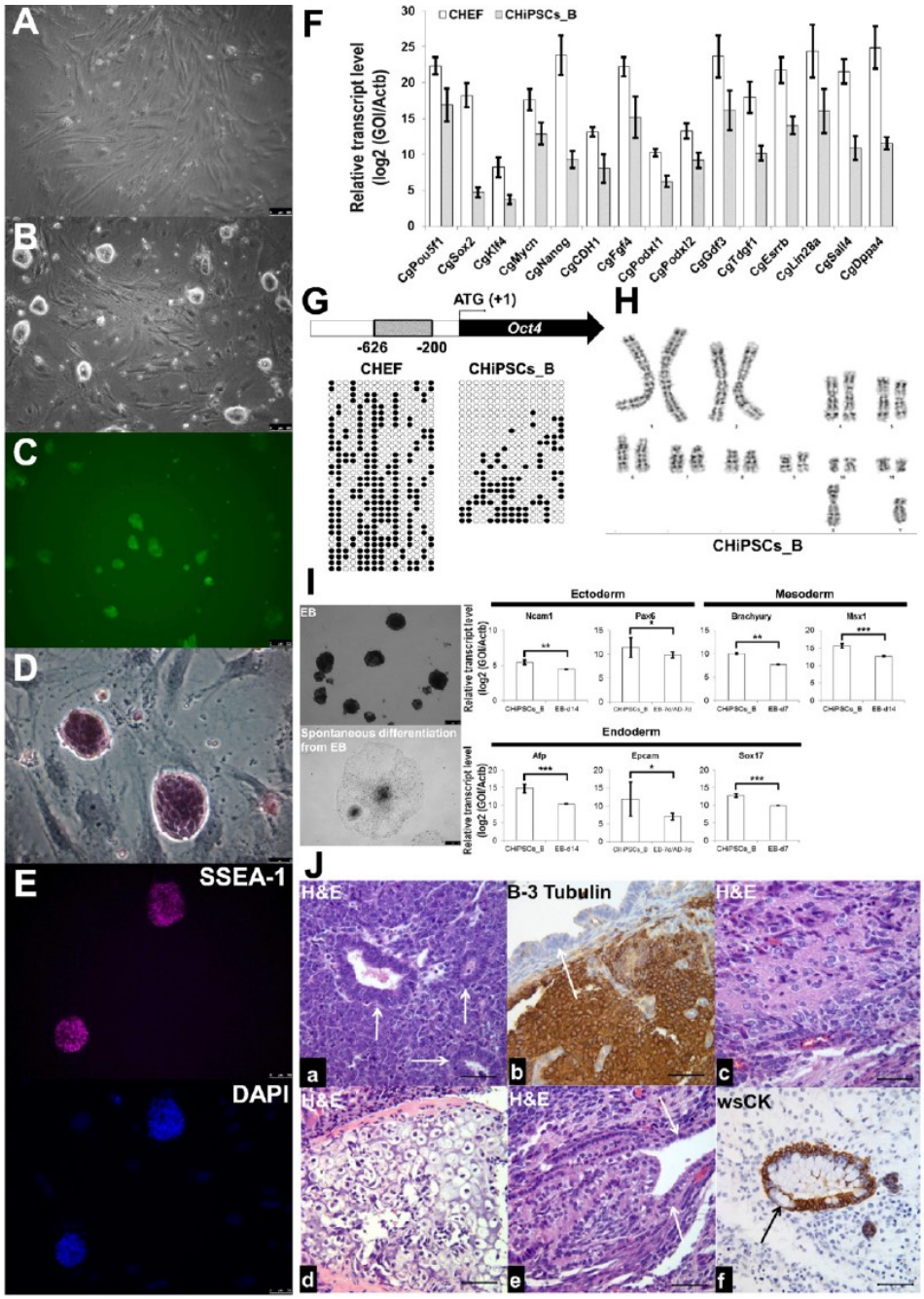

Fig. 1. Characterization of Chinese hamster-induced pluripotent stem cells (CHiPSCs) generated by lentiviral vector transduction. (A) Chinese hamster embryonic fibroblasts (CHEF). (B) Emergence of colonies. (C) Nanog-promopter GFP lentivirus infected cells of clone B. (D) AP staining of a colony from clone B. (E) Immunostaining of clone B for SSEA-1 and DAPI. Scale bar: 100 μm for A, B, and C; 25 μm for D; 50 μm for E. (F) Quantitative reverse transcription-PCR analyses of endogenous pluripotent marker transcripts in CHiPSCs-B. Data represented as mean ± SEM. $p < 0.001$. Cg in the label denotes that probes for the endogenous (Chinese hamster) gene were used. (G) Bisulfite genomic sequencing of the promoter region of Oct4. The open and closed circles represent a CpG site, representing unmethylated and methylated status respectively. (H) The CHiPSCs-B showed a normal 22,XY karyotype. (I) EB formed by CHiPSCs-B and the EB spreading on matrigel 7 days after plating for differentiation preceded by another 7 days of EB formation (Scale bar: 250 μm). qRT-PCR analyses of transcripts of differentiation markers of the three germ layers (Ncam1, Pax6, Brachyury (T), Msx1, Afp, Epcam and Sox17). Actin (Actb) transcript level was used as the reference. Data represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Histology of teratomas derived from CHiPSCs-B on (Ja) neurectodermal tissues comprised the majority of the teratoma and consisted of primitive neural tissues characterized by formation of neural rosettes (arrows) and cords of small polyhedral cells (surrounding rosettes) typical of neural progenitors which were (Jb) positive for β-3 tubulin (dark brown, IHC stain). (Jc) Other regions showed more mature central nervous system-like structures. (Jd) Multiple sites of cartilage differentiation were present which represent a mesenchymal lineage. (Je) Endodermal differentiation was demonstrated by the presence of tubular structures which were lined by cuboidal to columnar epithelial cells (arrows) and which were (Jf) positive for wide-spectrum cytokeratins (wsCK). The epithelium also contained cells with clear vacuoles consistent with mucus-containing goblet cells (arrow). Also noted was the absence of β-3 tubulin immunoreactivity in cells as seen in (Jb, arrow). Scale bar: 50 μm.

Table 1
Identification of Chinese hamster-induced pluripotent stem cells.

Classification	Test	Result	Data
Morphology	Photography	Similar to mouse ESCs: smooth, refractile defined colony borders and a compact internal architecture comprised of small, tightly packed round cells	Fig. 1B
Phenotype	NANOG expression: infecting CHiPSCs-B with a lentivirus wherein GFP is driven from a *Nanog* promoter	Cells had an active Nanog promoter	Fig. 1C
	AP staining	Positive for AP staining	Fig. 1D
	Immunocytochemisty	Positive for pluripotency markers: SSEA1	Fig. 1E
	The expression of endogenous pluripotency marker genes (qRT-PCR)	The expression of *Oct4, Sox2, Klf4,* and *c-Myc* were evident, additionally, *Gdf3, Tdgf1* (embryonic stem cell markers), *Esrrb, Lin28a, Sall4* (transcription factors), *Dppa4, Fgf4, Podxl1* and *Podxl2* (pluripotency) were all up-regulated in CHiPSCs-B compared to CHEF	
Epigenetic state	Bisulfite genomic sequencing of the promoter region of *Oct4*	The genomic segment from CHiPSCs-B was highly unmethylated (23.9% methylated) compared to CHEFs (47.5% methylated)	Fig. 1G
Genotype	Karyotype (G-banding)	A normal 22,XY karyotype	Fig. 1H
Differentiation potential	EBs formation	Transcript analysis showed that the EB formed from CHiPSCs-B expressed marker genes of all three germ layers, including *Ncam1* (ectoderm), *Pax6* (ectoderm), *Brachyury* (mesoderm), *Msx1* (mesoderm), *Epcam* (endoderm), *Afp* (endoderm) and *Sox17* (endoderm)	Fig. 1I
	Teratoma formation	Neurectodermal tissues comprised the majority of the teratoma and consisted of primitive neural tissues characterized by formation of neural rosettes and cords of small polyhedral cells typical of neural progenitors which were positive for β-3 tubulin. Other regions showed more mature central nervous system-like structures. Multiple sites of cartilage differentiation were present which represent a mesenchymal lineage. Endodermal differentiation was demonstrated by the presence of tubular structures which were lined by cuboidal to columnar epithelial cells and which were positive for wide-spectrum cytokeratins (wsCK). The epithelium also contained cells with clear vacuoles consistent with mucus-containing goblet cells. Also noted was the absence of β-3 tubulin immunoreactivity in cells	Fig. 1J

(Fig. 1C), alkaline phosphatase (AP) staining (Fig. 1D) and stage-specific embryonic antigen 1 (SSEA-1) (Fig. 1E). The expression of endogenous *Pou5f1, Sox2, Klf4,* and *c-Myc* were evident, additionally, *Gdf3, Tdgf1* (ESC markers), *Esrrb, Lin28a, Sall4* (transcription factors), *Dppa4, Fgf4, Podxl1* and *Podxl2* (pluripotency) were all up-regulated in CHiPSCs-B compared to CHEFs (Fig. 1F). The results show that the transcription of endogenous pluripotency genes was turned-on by reprogramming. A fragment of *Pou5f1* from −200 to −626 nucleotides from start codon containing 15 CpG sites was isolated from CHiPSCs-B and CHEFs for bisulfite sequencing. The fragment from CHiPSCs-B was highly unmethylated (23.9% methylated) compared to CHEFs (47.5% methylated) (Fig. 1G). Karyotype characteristics showed that the CHiPSCs-B had a normal karyotype of 22,XY in common with the CHEFs (Fig. 1H) (Kato and Yosida, 1972). To evaluate the differentiation capability, CHiPSCs-B was allowed to form embryo bodied (EBs) and to differentiate spontaneously by plating onto matrigel-coated culture dishes. Transcript analysis showed that the EBs expressed marker genes of all three germ layers, including *Ncam1* (ectoderm), *Pax6* (ectoderm), *Brachyury* (mesoderm), *Msx1* (mesoderm), *Epcam* (endoderm), *Afp* (endoderm) and *Sox17* (endoderm) (Fig. 1I). We injected CHiPSCs-B intramuscularly into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The resulting tumor contained various tissues corresponding to the three embryonic germ layers (Fig. 1J). Neurectodermal tissues comprised the majority of the teratoma and consisted of primitive neural tissues characterized by formation of neural rosettes (arrows in Fig. 1 Ja) and cords of small polyhedral cells (surrounding rosettes) typical of neural progenitors which were positive for β-3 tubulin (dark brown, IHC stain in Fig. 1 Jb). Other regions showed more mature central nervous system-like structures (Fig. 1 Jc). Multiple sites of cartilage differentiation were present which represent a mesenchymal lineage (Fig. 1 Jd). Endodermal differentiation was demonstrated by the presence of tubular structures which were lined by cuboidal to columnar epithelial cells (arrows, Fig. 1 Je) and which were positive for wide-spectrum cytokeratins (wsCK). The epithelium also contained cells with clear vacuoles consistent with mucus-containing goblet cells (arrow Fig. 1 Jf). Also noted was the absence of β-3 tubulin immunoreactivity in cells (Fig. 1 Jb, arrow).

Materials and methods

Cell culture

CHEFs were cultured in 45% EBSS medium, 45% RPMI 1640 Medium (Life Technologies™) supplemented with 10% fetal bovine serum (FBS, Hyclone). HEK293 cells were maintained in Dulbecco's modified eagle medium (DMEM, Invitrogen) containing 10% FBS. The CHiPSCs were maintained on feeder layers of irradiated *CF*-1 mouse embryonic fibroblasts (MEFs) (Evans and Kaufman, 1981). The cells were cultured in Knockout DMEM (GIBCO) supplemented with 20% Knockout Serum Replacer (KSR, GIBCO), a mixture of non-essential amino acids (GIBCO), 2 mM L-glutamine (GIBCO), 100 unit/mL penicillin, 100 μg/mL streptomycin (Cellgro), 0.1 mM β-mercaptoethanol (Invitrogen) and 2000 units/mL Esgro (LIF) (Chemicon).

Reprogramming

pLOVE series lentiviral expression vectors (Plasmid #15950, 15951, 15953), packaging (Plasmid #12260) and envelope expressing (Plasmid #12259) vectors were directly used in this study. The full-length mouse *Oct4* (*Pou5f1*) cDNA fused with the M3 domain of mouse *MyoD* at the amino terminus from pMXs-M_3O-IP vector was subcloned into the pLOVE-empty vector (Plasmid # 15948) for construction of pLOVE-M_3O. CHEF was then infection by lentivirus on day 0, and repeated on day 1, with 10 μg/mL polybrene (Millipore). On day 2 the medium was replaced with iPSC medium.

Table 2
Antibodies, kits, and primer sets used in this study.

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti- stage-specific embryonic antigen 1 (SSEA1)	1:100	R and D Systems Cat# MAB2155, RRID:AB_358058
Secondary antibody	647-conjugated donkey anti-mouse IgG	1:1500	Thermo Fisher Scientific Cat# A-31571, RRID:AB_162542
Teratoma analysis	Rabbit anti- β-3 tubulin antibody	1:500	Abcam Cat# ab18207, RRID:AB_444319
Teratoma analysis	Rabbit antibody against wide spectrum cytokeratin	1:4000	Dako Cat# Z0622, RRID:AB_2650434
Kits			
	Kits		Company
qRT-PCR reaction	RNeasy micro/mini Kit	Total RNA purification	QIAGEN
Bisulfite genomic sequencing	DNeasy Blood & Tissue Kit	Genomic DNA purification	QIAGEN
	EZ DNA Methylation-Gold™	Bisulfite modification	Zymo
AP staining	Leukocyte AP kit	Chemical staining	Sigma-Aldrich
Primers			
	Target	Forward/Reverse primer (5′-3′)	
Pluripotency Markers (qPCR)	*CgPou5f1*	*GATCAGCTTGGGTTGGAGAA/CGTTGGGTATAGTCGGTACTTG*	
Pluripotency Markers (qPCR)	*CgSox2*	*TGAAGGAGCACCCGGATTAT/TCCGGGAAGCGTGTACTTAT*	
Pluripotency Markers (qPCR)	*CgKlf4*	*GAGGAGCCCAAACCAAAGA/GGTGTGCCTTGAGATGAGAA*	
Pluripotency Markers (qPCR)	*CgMycn*	*GAAGATGACGAGGAGGAAGATG/CAGTGATGGTGAAGGTGGTAA*	
Pluripotency Markers (qPCR)	*CgNanog*	*GACTCTGCGTTTGTGGAGTAT/TCTGGTTGCTCCAAGTGTTAG*	
Pluripotency Markers (qPCR)	*CgCDH1*	*TATCCTTGATCCAGACCTTCCTCCC/TCACACACAATGATGTCCAGCGTGG*	
Pluripotency Markers (qPCR)	*CgFgf4*	*TTACAACGCCTACGAATCCTAC/CACAGTCTAGGGAGAAAGTGTG*	
Pluripotency Markers (qPCR)	*CgPodxl1*	*CCTTCTGAGATGCAGGAGAAA/CATCTAGGTCATCCTTGGTCAG*	
Pluripotency Markers (qPCR)	*CgPodxl2*	*GAGATTGGCATCCAGAACTACTC/GATCACCAGCACCACAAAGA*	
Pluripotency Markers (qPCR)	*CgGdf3*	*GCAGCAGACGGAAGAATTTG/GGCATGAAGAGAACGGATGA*	
Pluripotency Markers (qPCR)	*CgTdgf1*	*TCTGTCCTCCACGGTAACT/GACCATCACAGCCAGGTAAA*	
Pluripotency Markers (qPCR)	*CgEsrrb*	*CAAGAGGCTCAAGGTAGAGAAG/AGCCTCTAGGTCCTCAAAGTA*	
Pluripotency Markers (qPCR)	*CgLin28a*	*GAGGCGGTAGAATTCACCTTTA/GCTCACTCCCAATACAGAACA*	
Pluripotency Markers (qPCR)	*CgSall4*	*GGGCATGGACTCTATCCTTTAC/CATTGGTGTTGGCCACTTTG*	
Pluripotency Markers (qPCR)	*CgDppa4*	*AGGTACCAATTCCTCCTCTACC/TTGCTGCTCAGCTTCATCTC*	
House-Keeping Genes (qPCR)	*CgActb*	*GTCGTACCACTGGCATTGTG/AGGGCAACATAGCACAGCTT*	
Bisulfite PCR	*CgPou5f1* promoter	*TAAGGTTTGGAGGATTGAGTATGGAG/AAAAACTACRAACACCCTAAAAAAACAACC*	

Transduced CHEFs were subcultured onto MEFs on day 3 and colonies with compact ES-like cells were mechanically picked up between day 10 and 14.

AP staining and fluorescent immunochemistry

AP staining was performed using the Leukocyte AP kit. CHiPSCs were fixed with 10% formalin (Sigma-Aldrich) in PBS and stained with antibody against SSEA1 following 647-conjugated donkey anti-mouse IgG as secondary antibody, and Hoechst 33342 (1 mg/mL, Invitrogen) as nuclei counter stain.

Real-time quantitative reverse transcription PCR

Total RNA was prepared from cells using commercially available kits following the manufacturer's instruction. qRT-PCR was performed to analyze the expression of pluripotent genes in a Bio-Rad CFX Connect™ real-time PCR detection system using a Power SYBR® Green RNA-to-CT™ 1-Step Kit (ABI 4391178) (Table 1).

Bisulfite genomic sequencing

The genomic DNA samples were isolated and bisulfite-treated with commercially available kits (Table 2). After successful bisulfite PCR amplification of the *Oct4* promoter region (primers listed in Table 2), the amplicon was cloned into the pMD19 T-vector (Clontech). At least ten randomly selected positive clones from each sample (32 and 24 clones for CHEF and CHiPSCs-B, respectively) were picked-up and sequenced.

In vitro differentiation

To form EBs, CHiPSC-B was detached from culture plates as single cells by using 0.05% Trypsin-EDTA and transferred to an ultra-low attachment Petri dish in differentiation medium according to the previous report (Chang et al., 2009).

In vivo differentiation

Two-month-old NOD/SCID mice (Charles River) were intramuscularly injected with $1–4 \times 10^6$ cells for teratoma assay. After six to nine weeks teratomas were dissected and fixed with 10% formalin, then embedded in paraffin. Sections of 4-μm thickness were stained with haematoxylin and eosin solution.

Cytogenetic analysis

Cells were treated with colcemid, then harvested according to standard methods. The resulting metaphase cells were evaluated by G-banding. Minimum of 20 metaphases for each sample were examined with regard to chromosome number and structural rearrangements performed by the Cytogenomics Shared Resource in the University of Minnesota.

Acknowledgements

We thank Dr. Kyoungho Lee for his pLOVE-M3O vector construction. Histological analyses were performed at the University of Minnesota Comparative Pathology Shared Resource. The cytogenetic analyses were performed in the Cytogenomics Shared Resource at the University of Minnesota with support from the comprehensive Masonic Cancer Center NIH Grant #P30 CA077598.

References

Chang, H., Yoshimoto, M., Umeda, K., Iwasa, T., Mizuno, Y., Fukada, S., Yamamoto, H., Motohashi, N., Miyagoe-Suzuki, Y., Takeda, S., Heike, T., Nakahata, T., 2009. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. FASEB J. 23, 1907–1919.

Evans, M.J., Kaufman, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156.

Hirai, H., Tani, T., Katoku-Kikyo, N., Kellner, S., Karian, P., Firpo, M., Kikyo, N., 2011. Radical acceleration of nuclear reprogramming by chromatin remodeling with the transactivation domain of MyoD. Stem Cells 29, 1349–1361.

Kato, H., Yosida, T.H., 1972. Banding patterns of Chinese hamster chromosomes revealed by new techniques. Chromosoma 36, 272–280.